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(54) **Column agglutination assay and device**

Säulenagglutinationsassay und Vorrichtung

Essai d'agglutination en colonnes et dispositif

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(73) Proprietor: **ORTHO DIAGNOSTIC SYSTEMS INC.**
Raritan, New Jersey 08869-0602 (US)

(72) Inventors:
• **Hawk, Johnna B.**
Rocky Hill, NJ 08553 (US)

• **Chachowski, Rosemary K.**
Manville, NJ 08835 (US)
• **Setcavage, Thomas**
Milford, NJ 08848 (US)

(74) Representative: **Fisher, Adrian John et al**
CARPMAELS & RANSFORD
43 Bloomsbury Square
London WC1A 2RA (GB)

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EP-A- 0 104 881 EP-A- 0 194 212
EP-A- 0 305 337 FR-A- 2 660 437

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EP 0 485 228 B1

Description

This invention relates to the field of agglutination assays to detect the binding of ligands, and particularly immunological binding (antigen and antibody binding) such as that involved in blood group serology ("immunohematology").

Blood group serology requires the determination of blood cell compatibility between a blood donor and a patient recipient before a transfusion or organ transplant involving the patient. Blood cell compatibility is determined by the non-occurrence of an immunological reaction between antibodies contained in the blood serum of a patient and antigens present on blood cells from the donor.

Many different blood group antigens are found on the surface of the red blood cells of every individual. These antigens, the products of inherited genes, exist in a unique combination in everyone except identical twins. Blood grouping is generally the process of testing red cells to determine which antigens are present and which are absent, normally utilizing antibodies to the antigen tested for.

Additionally, when a person does not have a particular red cell antigen on his red blood cells, his or her serum may contain an antibody to that antigen. Whether or not the antibody is present in the serum depends on whether the person's immune system has been previously challenged by, and responded to, that specific antigen or something very similar to it. For example, a person whose red blood cells are Type A, i.e. having "A" antigens on the red cells, will have anti-B antibodies in his or her serum. Thus, if such a person is given type B blood, an immunological reaction will occur with possible serious clinical consequences.

As an additional consideration, it should be noted that the human body is constantly exposed to antigens in pollens, food, bacteria and viruses. Some of these "natural" antigens are apparently so similar to human blood group antigens that they stimulate almost every susceptible person to produce antibodies. Thus, certain antibodies are expected in the serum of anyone whose red cells lack the reciprocal antigen. This is especially true of the ABO system. Accordingly, a second confirmatory test is often performed on the patient/donor sera. The test for expected antibodies of the ABO blood group system in sera is called "reverse" blood grouping.

Antibodies of the ABO blood grouping system are generally immunoglobulin M (IgM). These antibodies have ten antigen binding sites per molecule. The IgM antibody is large enough to span the distance between red blood cells, so that when they are centrifuged, the cells will be bound together in a lattice "cell-antibody-cell-antibody" and will remain clumped together in agglutinates. For example, if anti-A is added to blood group A or blood group AB cells and the mixture is centrifuged, the cells will remain in clumps when resuspended. With the same antibody, group O and group B cells will resuspend as individual cells. Agglutination caused by one antibody, such as an IgM antibody, is called direct agglutination.

The anti-Rh blood group reactions tend to give weaker agglutination which can be enhanced by the addition of high molecular weight polymers. Some anti-Rh antisera consist of immunoglobulin G (IgG) antibodies and will cause direct agglutination, if facilitated by the presence of high protein concentrations, such as 25% bovine albumin (often found in commercial anti-D reagent preparations).

Such facilitation is needed as IgG cannot easily span the distance between cells which tend to repel each other. Thus, it will bind to red cell antigens matching its specificity, but will not agglutinate such red cells as effectively as the larger IgM antibodies will. The presence of IgG antibody bound to a red cell is thus usually detected by the addition of anti-IgG which will cause the requisite agglutination, resulting in a lattice of "red cell-IgG/Anti-IgG/IgG-red cell".

Serum naturally contains IgG that will neutralize the anti-IgG antibody added to bind to red blood cells. Therefore, the serum must be removed before such anti-IgG is added to the cells. Tests for IgG bound to red cells *in vivo*, are called direct antiglobulin tests. Tests for IgG bound to red cells *in vitro*, are called indirect antiglobulin tests. Such antiglobulin tests are also called "Coombs" tests.

It is standard bloodbanking practice to test for A, B and D (Rh₀) antigens on a sample of a person's blood (and to perform tests for other antigens in selected cases) and to crosscheck the results by testing the person's serum to determine the acquired antibodies that might be present. The former is referred to as "forward typing", while the latter is referred to as "reverse typing". The results from each of these typing exercises have to agree.

Since the early 1900's, the general approach, known as the "Landsteiner" method, has been to add a patient's red blood cells to a standard laboratory test tube containing a blood group antibody (such as Anti-A or Anti-B), mix to allow antibody/antigen binding reactions to take place, and then to centrifuge. If the antigen tested for is present, antibody/antigen binding will have taken place, resulting in agglutination of the patient's red blood cells. The test tube is manually shaken to dislodge the centrifuged button of "clumped" cells at the bottom. A subjective determination is then made as to whether or not the dislodged cells are "clumped", and to what extent.

During the mid-1900's, attempts were made to simplify this technique to minimize the subjective nature of the test and to reduce mistakes. It was recognized that a somewhat permanent record of the results of compatibility testing could be had by employing wettable, either non-absorbent or, in some cases, absorbent test slides or test cards having the requisite immunochemical reagents on at least a portion of their surfaces. U.S. Patents nos. 2,770,572, 2,850,430, 3,074,853, 3,272,319, 3,424,558, 3,502,437 and 3,666,421 and European Patent Application no. 0 104 881-A2 depict

select examples of such test cards and related apparatus.

More recently, techniques to increase the sensitivity and accuracy of certain antiglobulin testing, such as the so-called "Coombs" test, have been developed. U.S. Patent No. 4,435,293 to Graham et al. describes a bloodtyping system (Simwash[®]) which uses a system of test tubes that eliminates the washing steps of the original Coomb's method, as the Simwash[®] system provides "self-washing" of the red blood cells. Antiglobulin tests, such as that just described, require that the red blood cells be free of their serum, which contains unbound IgG antibodies. With the Simwash[®] system, the dense cells are centrifuged into a column, while the less dense serum remains at the top.

European Patent Application no. 0 194 212 describes a blood compatibility testing system using gel in a column, such gel being in particular, Sephadex[®] which is a 3-dimensional network or matrix of dextran chains crosslinked with epichlorhydrin (product of Pharmacia Fine Chemicals, Uppsala, Sweden and Piscataway, N.J.), that catches agglutinated red blood cells and allows unagglutinated cells to pass through to the bottom.

One of the drawbacks in the use of the last-mentioned system, and especially in the manufacture of such a test system using gel as a medium, is that gels such as Sephadex[®] have to be settled prior to insertion into a test tube to get rid of the so-called "fines", which are contaminating small molecular weight compounds or fragments that may interfere with the separation capacity of the gel. Gel fines have historically been known to clog chromatographic separation columns.

A gel also has to be swelled prior to use and in order to accurately calculate the amount of reagents to be used in conjunction with the test system. Calculations of requisite amounts of reagents, buffers and the like have to take into account the gel swelling dilution factor. Additionally, some of the reagent is lost as it is absorbed by the porous gel and not available for binding to the analyte tested for. Greater quantities of reagent must be added to compensate for this loss. The gel is also inherently more fragile to mechanical handling, and can break apart during the swelling process, causing an uncontrollable variation in gel particle size.

Another drawback one suffers in the use of Sephadex[®] is that it is very fragile to temperature extremes, and tends to dry out or break apart, causing stability, shipping and storage problems, which include shrinkage, and concurrent alterations in test properties. The breakage can result in such above-mentioned fines that alter or restrict the normal flow of the blood cells through the material. An additional shipping and storage problem is occasioned by the inability to freeze Sephadex[®]-containing test devices.

European Patent Application No. 0 305 337 describes a variation of the device described above. It shows the use of a gel column, but contained in a reaction vessel which narrows via a taper around its mid point.

The present invention provides a device for trapping agglutinates as defined in claim 1. The present invention also provides a method for detecting the binding of ligands as defined in claim 7. Preferred features of the device and method of the present invention are defined in the dependent claims.

The present invention provides a method and device for detecting the presence of binding ligands, especially blood group antigens or antibodies, which provides superior performance in allowing movement of non-agglutinated reactants, especially red blood cells, while constraining, preferably via so-called "band formation", agglutinated reactants, especially agglutinated red blood cells.

The microparticles may be impregnated with or immersed in a reagent to detect binding ligands such as blood group antigens or antibodies, prior to the addition of the matrix to the device. Alternatively, a liquid form of such a reagent may be added to the opening of the container so that it may filter through the microparticles contained therein. A liquid sample possibly containing an analyte of interest that will bind to the reagent and cause an agglutination reaction is also added to the container, either concurrently with the mentioned reagent, or subsequent to the addition of the reagent. The container is centrifuged and the presence or absence of agglutinates detected.

The following is a more detailed description of certain key aspects of the invention. The preferred device of the invention is generally a container, or elongate substantially transparent hollow member, having at least one open end, sometimes referred to herein as an inlet port. By inlet port is meant an area to which samples and/or reagents, buffers, and the like may be applied to eventually achieve contact with the matrix of the invention. In some embodiments, the elongate member has a substantially closed end, opposite the inlet port. By substantially closed end is meant closed to such an extent that the matrix is retained within the member, and does not spill out, and reactants one wishes to retain in the matrix are also retained. By elongate is meant a container that is somewhat longer in proportion to its width and aligned along a longitudinal axis. The matrix of the invention is thus housed in a somewhat columnar fashion so that reactants may move through its length if nonagglutinated, and be caught by the matrix if agglutinated. A container shape that is long enough in the area that houses the matrix to allow this separation and detection thereof, will be suitable for the purposes of the invention, it being understood that the remaining aspects of the container shape may differ considerably. The area of the hollow member or container that houses the matrix is generally referred to herein as the "agglutinate detection zone."

The overall shape of the container should, in general, facilitate this separation process. For example, in preferred embodiments, the entire container is basically of a laboratory test tube shape, including those test tubes that may be described as "cone-shaped" in their entirety or a portion thereof. Other preferred embodiments include those config-

urations wherein only a portion of the container is cone-shaped, (e.g. in the area that contains the matrix). In this case, the cone-shaped area may be joined to a sample-receiving area that is of a shape suitable to contain the samples or reagents as added by the user, and may be rounded, cuboidal and the like in configuration. By way of example only, Figures 1-4 depict various shapes and configurations of portions of devices for carrying out the method of the invention.

A housing having two separate areas, each defined by its relevant shape, provides the additional advantage of an "upper" chamber for incubation of cells and serum prior to movement of this mixture through the matrix. This is especially true when the juncture between this upper chamber (sample-receiving area) and the agglutination detection zone (or area just above it) is defined by an opening that is smaller than the opening to which samples and reagents are applied (the latter being the inlet port area). This is because the larger inlet port opening facilitates the adding of cells and serum, while the smaller opening prevents their passage through the matrix until the user applies a force, such as centrifugation, to effect their movement. The user may therefore exert control over the timing of the agglutination assay. Figures 2 and 3 illustrate this concept. Figure 2 depicts a housing 80 with an upper chamber 95 disposed therein for receiving samples, said upper chamber having an opening 105 which will not permit movement of samples applied to the upper chamber until the user effects that movement in an appropriate manner. Figure 2 also depicts an "initial reaction zone" 103 containing buffer or a reagent for the binding and agglutination steps of the assay. A band of agglutinated reactants 100 is formed within the matrix 90. Figure 3 is an alternate embodiment which lacks the initial reaction zone. In this embodiment, the binding reaction may have been performed prior to addition of the reactants to the device, with the matrix acting as a filter for the agglutinates. Alternatively, the reactants may have been added directly to the upper chamber and allowed to react for a time prior to centrifugation and passage to the matrix below. The agglutinates are then caught on top of or in the matrix, after centrifugation through the opening 105. Figure 4 depicts yet another configuration for the agglutination detection zone of the device and also depicts formation of the band 100 within the matrix rather than on top of the matrix.

By "substantially transparent" is meant any material that is translucent or transparent to such a degree that the presence or absence of agglutinates may be readily ascertained either by the naked eye or through the use of detecting instrumentation for that purpose. The device may be substantially transparent in its entirety or only in selected areas as, for example, the area of agglutinate band formation in a positive sample. It is within the contemplation of the invention that observation of positive and negative agglutination reactions may be made with an instrument designed to optically or otherwise detect cluster, agglutinate, band and/or button formation within a certain area of the agglutination reaction zone. This observation process may be performed manually with the instrument or it may be a so-called "user walk-away" automated observation process.

Containers such as laboratory test tubes and columns generally known to the art of immunoassay and bloodbanking may be used for the purposes of carrying out the method of the invention, as long as they are adapted to centrifugation and instrumental observation, if desired. Suitable containers may comprise materials such as glass, polystyrene, polyethylene, polypropylene, polycarbonate and the like. Preferred is the test tube cartridge, which is generally a premolded rectangular-shaped cartridge containing several tubes. Figure 1 depicts one embodiment of such a cartridge. Especially preferred are the dual chamber laboratory test tubes such as those described previously, particularly if such test tubes are in cartridge form and adapted to be filled with reagents, buffers, matrix and the like with automatic pipetting devices, during the manufacturing of the devices or later when the devices are used to conduct assays.

The matrix of the invention comprises substantially noncompressable, acid washed, non-porous glass microparticles. By "substantially noncompressable" is meant resistant to change in shape or size that may be caused by the exertion of force to the matrix, such as centrifugal force, magnetic force, electrical force, hydrostatic pressure, force by negative or positive pressure and the like, or storage for long periods of time with normal gravitational force. The particles may be of any shape as long as the movement of unagglutinated reactants is not impeded by irregularities, and so on. The size of the particles may vary considerably according to the particular binding ligands involved in the agglutination assay. One skilled in the art will understand that agglutinated reactants should be retained in or on top of the matrix while non-agglutinated reactants travel through the matrix to the bottom or outside of the device altogether. However, in the case of agglutination of red blood cells, preferable matrix microparticle size ranges are generally from 50 μm to 300 μm , and more preferably 50 μm to 200 μm , and most preferably 50 μm to 150 μm .

The matrix as described above is provided in the agglutinate detection zone of the device of the invention. If the device is columnar in shape, the matrix is most often provided along its length proximate to a closed end. However, in some embodiments, the matrix is provided only throughout a "middle" portion of the device and may even, in some embodiments, rest on top of a second material serving as a plug, such as a glass fiber or other type of fiber. The devices may also interface with instrumentation at one or more locations. For example, the inlet port may be joined to an instrument, such as a pipetting device to receive samples therethrough. The device may interface with instrumentation designed to effect movement of the reactants, for example to push or to pull them through the matrix. In some embodiments, a vacuum may be applied to pull reagent liquid and even nonagglutinates through the device, while agglutinates are retained within or on top of the matrix. In other embodiments, instrumentation may interface with a portion of the device to apply a force, such as a liquid stream, to move the reactants through the matrix.

Prior to adding the matrix microparticles to this zone, they may be first washed in any appropriate manner to remove undesirable contaminants, and to avoid nonspecific binding. The particles may be added to the device in a slurry consisting of particles in antiserum, buffer or other desired reagents or diluents, whether alone or in combination. The particles may also be added to this zone in a dry form and antisera, appropriate buffers and the like added subsequently, if desired. In preferred embodiments, the microparticles are completely immersed in an appropriate reaction solution or buffer within the agglutinate detection zone of the housing and a layer of the solution alone extends from a top portion of the matrix. This extended area is referred to as the "initial reaction zone" and in columnar devices other than the dual chamber devices, is an area wherein reactants may first come into contact with each other, mix and begin to react.

By way of illustration, the column agglutination method of the invention will be described in the context of certain bloodtyping and compatibility procedures. A forward bloodtyping assay may be conducted utilizing the column agglutination device as herein described by using a columnar device to which has been added the matrix of the invention. A monoclonal antibody or polyclonal antibody containing antiserum is dispersed in a physiologically compatible buffer and added to the matrix of microparticles to immerse them and extends from the matrix toward the inlet port to form the initial reaction zone. Suitable amounts of such antibodies may be routinely optimized by those skilled in the art, depending generally on the antigenic affinity and specificity of the antibodies. The antibodies are dispersed in a buffer that may also contain suitable additives known to the art to help potentiate their reactivity and prevent nonspecific binding, such as high molecular weight polymers and the like. Examples of these include polyvinyls, dextran, gelatin and polyethylene glycol. Lower molecular weight polymers may also be added to increase the density of the solution.

To the initial reaction zone is added a saline suspension of a patient's red blood cells. Those skilled in the art will readily ascertain that a suitable suspension of cells is 2-6%, and preferably 2-3%. If the suspension is too dilute, any resulting agglutination reaction will be difficult to read. If the suspension is too concentrated, the system will be overloaded and agglutination indistinguishable. The binding reaction is allowed to take place and then movement of the reactants to and through the matrix is effected. This movement is generally effected by exerting a force, such as those forces described above. In certain preferred embodiments, a centrifugal force, negative pressure or a hydrostatic force is applied.

If centrifugation is the technique utilized to exert force on the reaction mixture to effect its movement onto or through the matrix, then one skilled in the art will appreciate that centrifugation times and speeds may vary greatly for optimal results. In preferred embodiments, centrifugation should be at a speed and for a time suitable to allow the agglutinates, if any, to form a cluster or band near the top portion of the matrix column, rendering a clearly observable positive reading, while the non-agglutinated negative cells travel to the bottom of the device to form a button. If the device is spun for too long or at a speed that is too high, the agglutinates will be forced to the bottom of the tube. It is also desirable that a centrifuge with a swing-out head be utilized to ensure that the reactants travel somewhat through the center of the matrix area and do not lie on the side of the device.

In this illustration, if antibodies to Type B blood cells were added, Type B blood cells contained in the patient's sample will bind to the antibody, forming a line of aggregated cells trapped near the top portion of the matrix. Type A cells will not agglutinate and will be spun to the bottom of the device. Often times, a patient's cells may contain weakly reacting variants. The moderate reaction might be demonstrated by smaller scattered agglutinates throughout the column matrix, while negatives form a button on the bottom of the matrix column. Figure 1 demonstrates examples of reaction end-points that may be observed using the method and device of the invention. Tube 10 demonstrates a strong positive reaction, with a firm band 100 of agglutinates. Tube 12 shows a positive reaction that is somewhat weaker than the previous, as the agglutinated band has broken apart into smaller agglutinates. Tube 14 demonstrates a more moderate positive reaction with smaller agglutinates distributed throughout the middle portion of the matrix, and even settling to some extent on the bottom, as in tube 16. Tube 18 depicts a very weak positive reaction, with most of the cells collected on the bottom. Tube 20 shows a clear negative, with a button of cells 50 on the bottom of the tube and no agglutinates dispersed within the matrix 90.

The device and method of the invention may be utilized for direct agglutination studies of the kind just described for the detection of many different blood cells including white blood cells, platelets, red blood cells and the like. If visual observation of agglutinates is desired, it is preferable to first stain any colorless cells or colorless particles adhering to cells, with a dye suitable to effect a visually perceptible agglutination reaction.

The hemoglobin in red blood cells provides such a suitable color, naturally, without staining. Direct agglutination studies may be performed on ABO red blood cells as illustrated above, as well as those blood cells containing D, C, c, E, e, K antigens and the like. Similarly, when sera are to be tested for the presence of antibodies to a particular antigen or cell containing an antigen, they can be mixed with known antigens. If the unknown serum contains antibodies to the known antigen that is provided, the reactants will be agglutinated and trapped when they move onto or down through the matrix, while negative serum will not effect a reaction, and agglutinates will not be trapped. It is preferred to utilize a dual chamber configuration in conducting these last-mentioned assays to allow the known antigen or cells containing antigen to incubate with the patient's serum prior to movement of the mixture onto or through the matrix by

centrifugation or other appropriate means.

In the context of immunohematology applications of the device and method of the invention, a patient's serum may also be screened for unexpected antibodies in the manner described above, such as the Kell, Duffy and Kidd antigens. Antiglobulin tests such as the Coombs Test may also be performed. In Coombs testing, the cells should be free of serum containing unbound antibodies. With the system herein described, the dense cells are centrifuged into the column. The less dense serum remains at the top of the column similar to the process disclosed in U. S. Patent no. 4,435,293. Anti-IgG dispersed within the matrix will agglutinate cells which have IgG antibodies bound to their antigens and the agglutinates will be trapped on top of or in the matrix. Cells which have no IgG bound will travel through the column to the bottom of the tube.

The device and method of the invention have been illustrated in great detail for use in a blood serology context. However, it should be understood that it is within the contemplation of the invention to conduct binding assays involving any binding ligands associated with particles, such that the particles will agglutinate as a result of the binding of the ligands to their binding partners. For example, any binding ligand that could be attached to a particle or other carrier that takes the place of the red blood cells (bearing antigens) might be suitable. The particles serve as carriers for these ligands just as the red blood cell "carries" its antigen. Examples of other ligands include specific binding proteins disclosed in U.S. Reissue Patent No. 31,006 to Schuurs et al. Such particles might be inert carriers such as those disclosed in U.S. Patent No. 4,140,662. Suitable carrier particle size and composition will vary according to the binding ligands one seeks to detect, examples of which are disclosed in the referenced patent. Suitable matrix microparticle size and composition will also vary in accordance with the selected carrier particles and binding ligands. The examples section of this specification provides guidance in techniques useful to determine optimal particle size and composition. Although a red blood cell agglutination system is illustrated, one skilled in the art will appreciate that other systems may be optimized in this manner. In some embodiments, it is preferable that the particles be colored, to enable a visual observation of agglutinated clumps or bands of particles, similar to the effect obtained with the visually colorful red blood cells.

The claimed device and method avoid the drawbacks associated with devices and methods known in the art of agglutination assays, by providing an assay system that utilizes a matrix of substantially noncompressable microparticles. Such particles do not have to be "swelled" prior to use in this system and prior to calculation of amounts of reagents to be added. The particles are less porous than other matrices known to the art and accordingly do not absorb a great deal of any reagents that might be added, which renders more of the reagent available for reaction. Also, variation in particle size is minimal as a great deal less breakage occurs. Surprisingly, the assay system of the invention requires less centrifugation time, when centrifugation is employed to move the reactants through the matrix.

These factors, inter alia, result in an assay system that tends to require less reagent and is therefore quite cost effective. Additionally, the system provides the added advantage of greater storage and shipping capabilities. For example, if the devices happen to turn upside down in the shipping or storage phase, the matrix may be easily tapped back into place within the support member. Also, the devices containing the matrix of the invention may be stored in a frozen state, 15°C, or at room temperature, without stability problems.

The following specific examples exemplify the invention, but should not be considered as limitative thereof.

I. MICROPARTICLE SELECTION

The following experiments depict a selection of substances which will allow unagglutinated red cells to pass through a column when centrifuged, while agglutinated red cells are trapped either on top of or in the column. The general methods for conducting the following experiments were:

1. particles added to a container, such as a test tube;
2. red blood cells under test are mixed with reagent containing antibodies and then deposited onto matrix of particles;
3. the tubes are centrifuged in a centrifuge capable of spinning containers horizontally;
4. the tubes are observed for agglutination/nonagglutination.

A. Testing Various Particles:

Small plastic tubes were prepared with about 1/2 ml microcellulose (Whatman Microcellulose DE32, DE52, QA52, SE52 (prefixes indicate different charges)). (volume is packed volume - from slurry made in phosphate buffered saline (PBS))

EP 0 485 228 B1

Results: DE32/cells do not pass through DE52/some cells in top 1/2 of tube, others pass to bottom

Resuspended in anti-human globulin (AHG); then added IgG coated cells. (Ortho Coombs Control Cell™ e.g. OCC) Control and negative cell (Selectogen™).

DE52

QA52 All negative cells passed to the bottom

SE52

Tested Aerosil = approximately 740nm diameter silicon dioxide particles and Dow 0.8μ polystyrene latex particles

Cells did not go through these suspensions even after a hard spin.

Discussion:

Human red cells (7μm diameter) can be centrifuged through a bed of particles of 100-150 μm.

B. Tested additional particles in test tube cartridge:

Cellulose particle sizes are much larger (≈100-150μm) than red cells (7.0μm).

1. Cellulose Whatman C F11 - fibrous cellulose powder

2. Cellulose whatman C F31 - microgranular

3. acid washed glass beads - less than 150μm

- 150-212μm

- 425-600μm

Procedure

(1) Particles are suspended in Ortho Bioclone

Anti-A™ lot # BAA 1100

(2) suspension loaded into upper portion of device.

(3) Centrifuged lightly - cellulose stuck in top.

Plug broken with probe and respun to load cellulose in bottom.

The glass beads may be loaded dry and liquid added.

(4) 10ml Affirmagen™ A cells or B cells were added

(5) Centrifuged in a table-top swing-out head centrifuge (Sorvall GLC-1 microtiter plate carrier), 900 RPM for short periods to find time required for B cell to reach bottom.

	30'		Plus 1 min.		Plus 1 min.		Plus 5 min	
	-	+	-	+	-	+	-	+
Cellulose 1	T	T	1/2	T	1/2	T	1/2	T
Cellulose 2	T	T	T	T	T	T	1/10	T
Beads (S) 3	part	T	B	T	B	T	B	T
Beads (M) 4	part	T	B	T	B	T	B	T
Beads (L) 5	part	T	B	T	B	T	B	T

T = cells on top

B = cells on bottom

Part or fraction refers to cells on bottom.

All sizes of glass beads tested worked very well for typing A cells- spin time between about 30-90 seconds.

Tried large bead (425-600μm) in commercial anti-IgG with a strongly coated cell (Ortho Coombs Control Cell™ (OCC)) and an uncoated cell (Affirmagen™ A cell)

25μl AB serum; 10μl cell suspension; spun 1 minute 900 rpm.

Separation of cells from serum allowed antiglobulin to agglutinate cells. Agglutinates were trapped in the column of beads.

Discussion:

The above section B studies were a continuation of the selection of particles which will allow unagglutinated cells to be centrifuged through while trapping agglutinated cells. Two cellulose powders and three glass bead sizes were tested. They were each suspended in commercial blood grouping serum Anti-A. Red cells of blood group A or B were added and carefully centrifuged through the columns of particles being tested.

Tests for IgG antibodies in human serum are preferably performed with the indicator cells separated from the test serum, since the serum contains relatively large amounts of IgG which would neutralize the reagent anti-IgG. Cells known to be coated with IgG antibodies were mixed with group AB serum known not to contain antibodies to the cells. Cells known to be not coated with antibodies were tested as a control. The mixtures were added to the top of columns of glass beads in commercial anti-IgG reagent then centrifuged.

Results

Some but not all of the negative cells were centrifuged through the cellulose particles. The agglutinated cells remained on top of the column. All of the negative cells were at the bottom of the glass bead columns and all of the positive cells remained at the top.

Cells coated with IgG antibodies were agglutinated by the Anti-IgG trapped in the column of beads. Cells not coated with IgG antibodies were not agglutinated and were at the bottom of the column. There was a marked difference seen between negative and positive examples when anti-IgG was used.

The test sera and cells used for these experiments were selected as those which give strong reactions. The glass beads provided support for agglutinated A cells and unagglutinated B cells were easily spun through the columns. The antibody coated cells were separated from the serum in which they were suspended at least well enough to detect strong reactions. Serum remains in the upper portion of the column and does not neutralize the anti-IgG.

C. Additional Particles:

Iron metal filings, ≈ 40 mesh obtained from Fisher 157-500.
Sea Sand, washed; obtained from Fisher-S25-500, lot901223
Sea Sand, washed; and dried; obtained from Mallinkrodt 7062 - lot 7062 KED2.

1. Added 5 to 7 mm of particles under test to each of 2 columns in a test tube cassette.

2. Added Ortho Bioclone Anti-A™, lot BAA110D to each column, mixed to get air pocket out. Iron filings packed in such a manner that the needle used for stirring would not go through the filings or down the side. Some bubbles of air remained in the bottom of the tube.

3. Added 10 μ l of cells to each (Ortho Affirmagen Anti-A™, lot #A844). A cell is positive and B cell is negative with Anti-A.

4. Centrifuged for ≈ 8 min. in table-top swing-out head centrifuge.

Results:

Iron filings look black, cannot see red cells. Need to use filings that will be more appropriate as a background color for the color of test particles under study (i.e. red cells). Fisher sand and Mallinkrodt sand both held agglutinates on top, both sands allowed the negative cells to pass through to the bottom.

Mallinkrodt sand was whiter and provided a superior background color for assessing a negative reaction.

Discussion:

In the above section C studies, additional solid particles were screened to find alternative support media. Iron metal filings and two sources of sea sand were tested using blood grouping serum anti-A with A and B blood cells. The sands were tested in indirect antiglobulin tests. Twofold dilutions of anti-D were tested to determine sensitivity.

Iron filings were eliminated from testing because the dark color obscured the red cells. Both sea sands clearly

trapped A cells on a column containing Anti-A and allowed the B cells through. After adjustment of centrifugation and column length the sensitivity of the antiglobulin test was found to be comparable to standard manual test results.

The sand samples used in the previous study were soaked in acid cleaning solution (dichromate) overnight. The sands were rinsed in tap water, and then distilled water, and placed in oven to dry. The particles were then examined under a dissecting scope.

Iron filings - all were rough with jagged edges. Particle size varied greatly from about 2 mm to about 200 mm.

Fisher sand - particles from bottle are charged and repelled by a spatula.

Appearance: 100-150 μ m average size (some are smaller); clear quartz.

Mallinkrodt sand - more rounded than the Fisher specimens; translucent surface; probably beach tumbled.

Fisher acid-washed sand is tan with dark flecks. Mallinkrodt is much whiter with fewer flecks.

Tested acid washed sand vs sand from the bottles (both labeled "washed") with Bioclone Anti-A™ and preliminary test with Anti-Human serum. The acid washed looked cleaner - no other difference noted - no cells appeared to be bound to sand.

D. Addition of High Density Solution:

Coombs Test:

Anti-IgG 0.5% PEG, 2% Dextran 87000MW.

Tested AB serum lot 124B40 - 40 μ l

Ortho Antibody Enhancement Solution™ (low-ionic) lot AES 204-140 - 40 μ l
10 μ l Coombs Control Cell™ K430

Anti-human globulin cells seem to be sticking to sand. Cell button at bottom is somewhat larger with A cell than in the OCC.

Tested with beads in normal rabbit serum (NRS) pool and PBS dilutions of normal rabbit serum using Affirmagen™ A cell and OCC cells (IgG coated), in a test tube cartridge.

These were spun in horizontal tabletop centrifuge for 9 minutes, then in a Sorvall GLC 900 RPM for 5 minutes. The matrix appears pink stained. This might be due to non-specific adherence.

Mallinkrodt sea sand with undiluted NRS looked the best, as fewer cells adhered to the sand. However, cells are agglutinated by an antibody in the undiluted NRS.

Tested Mallinkrodt sand anti-IgG in 5% PEG K20

2.5% Dextran, \approx 20K MW

0.1% gelatin in PBS

40 μ l serum		controls		serum OAES	
40 μ l OAES		10 μ l cells OAES		5 μ l cells	
-	+	no serum	-	+	-
A cell	OCC	+	-	+	-
		OCC	A cell	OCC	A
cell					

A definite difference was observed between + and -. The second two sets were spun 5 minutes in a Sorvall GLC at 1000 RPM.

Repeated testing of Mallinkrodt sand. The second two sets 10mm column

Spin GLC 2000 RPM 5 minutes

anti-IgG as before, cells OCC(+) and A(-)

#1 Control	#2 Test
10 μ l cells	40 μ l serum
	40 μ l OAES
	10 μ l cells

Results: Positive agglutinated and negative spun-down. This demonstrates that can separate the serum from the system, and get a positive reaction.

Tested anti-D diluted in serum - as above, in the following dilutions:

1/100	1/100	1/1000	1/2000	1/4000	0
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Test results are comparable to conventional anti-human globulin manual testing (indirect Coombs). 1/1000 is a definite positive reaction; 1/2000 looks like agglutination at the bottom of the tube; 1/4000 may be slightly agglutinated; the negative has a rounded button of cells at the bottom of the tube.

The object of this experiment was to determine whether a high density solution such as the dextran albumin solution used in the Simwash Serum/Cell Separation System^R would improve the column agglutination antiglobulin test. Anti-IgG was diluted in Simwash^R solution and albumin added. Cells coated with IgG were added and cells not coated served as a control.

Results

The coated cells were agglutinated and trapped by the beads. Some of the uncoated cells bind to the glass beads.

Discussion

The use of a higher density medium provides a better separation of serum and cells, especially useful in performing Coombs tests.

Claims

1. A device for trapping agglutinates comprising:

a substantially transparent hollow support member having an inlet port; and
a matrix of substantially non-compressible, acid-washed, non-porous glass microparticles contained in said support member, wherein:
said matrix permits movement of non-agglutinated reactants but does not permit significant movement of agglutinated reactants therethrough; and
there is a sufficient quantity of said matrix in said support member to allow separation of agglutinated reactants from non-agglutinated reactants to be observed visually.

2. The device of claim 1, wherein said matrix is covered by a solution containing reagents.

3. The device of claim 2, wherein said solution has a high density.

4. The device of claim 2 or claim 3, wherein said solution contains antibodies.

5. The device of claim 4, wherein said antibodies are IgG antibodies.

6. The device of any one of claims 1 to 5, wherein said microparticles range in diameter from 50 to 150 μm .

7. A method for detecting the binding of ligands comprising the steps of:

(a) applying a sample possibly containing a binding ligand to a device according to any one of claims 2 to 6 wherein said reagents include a corresponding binding partner for said binding ligand;
(b) applying force to said sample to cause said sample and said solution to mix so that any binding ligand present in the sample binds to its corresponding binding partner, thereby to form an agglutinate, and to cause unagglutinated reactants to pass through said matrix and agglutinated reactants to be trapped on or within said matrix; and
(c) detecting the presence or absence of agglutinates on top of or within said matrix.

8. The method of claim 7, wherein said force is applied by centrifugation.

Patentansprüche**1. Vorrichtung zum Abfangen von Agglutinaten, umfassend:**

ein im wesentlichen durchsichtiges, hohles Trägerelement mit einer Einlaßöffnung; und eine Matrix aus im wesentlichen nicht-komprimierbaren, mit Säure gewaschenen, nicht-porösen Glasmikroteilchen in dem Trägerelement, wobei:
die Matrix eine Bewegung von nicht-agglutinierten Reaktanten erlaubt, jedoch keine signifikante Bewegung von agglutinierten Reaktanten durch die Matrix erlaubt; und
wobei eine ausreichende Menge der Matrix im Trägerelement vorhanden ist, um eine Trennung von agglutinierten Reaktanten von nicht-agglutinierten Reaktanten zur visuellen Beobachtung zu ermöglichen.

2. Vorrichtung nach Anspruch 1, wobei die Matrix mit einer Lösung mit einem Gehalt an Reagenzien bedeckt ist.

3. Vorrichtung nach Anspruch 2, wobei die Lösung eine hohe Dichte aufweist.

4. Vorrichtung nach Anspruch 2 oder 3, wobei die Lösung Antikörper enthält.

5. Vorrichtung nach Anspruch 4, wobei es sich bei den Antikörpern um IgG-Antikörper handelt.

6. Vorrichtung nach einem der Ansprüche 1 bis 5, wobei die Mikroteilchen in einem Durchmesserbereich von 50 bis 150 µm liegen.

7. Verfahren zum Nachweis der Bindung von Liganden, umfassend folgende Stufen:

(a) Auftragen einer Probe, die möglicherweise einen bindenden Liganden enthält, auf eine Vorrichtung nach einem der Ansprüche 2 bis 6, wobei die Reagenzien einen entsprechenden Bindungspartner für den bindenden Liganden enthalten;

(b) Anlegen einer Kraft an die Probe, um eine Mischung der Probe und der Lösung herbeizuführen, so daß der in der Probe vorhandene bindende Ligand an seinen entsprechenden Partner bindet, wodurch sich ein Agglutinat bildet, und um zu bewirken, daß nicht-agglutinierte Reaktanten durch die Matrix gehen und agglutinierte Reaktanten an oder innerhalb der Matrix eingefangen werden; und

(c) Nachweisen der Anwesenheit oder Abwesenheit von Agglutinaten auf der Oberseite oder innerhalb der Matrix.

8. Verfahren nach Anspruch 7, wobei die Kraft durch Zentrifugation angelegt wird.

Revendications**1. Dispositif pour la capture d'agglutinats, comprenant:**

un élément de support creux pratiquement transparent, comportant un orifice d'entrée; et une matrice de microparticules de verre non poreux, non comprimables, lavées à l'acide, contenues dans ledit élément de support,

dans lequel:

ladite matrice permet le mouvement de corps en réaction non agglutinés mais ne permet pas un mouvement notable, dans cette dernière, des corps en réaction agglutinés; et

une quantité suffisante de ladite matrice est présente dans ledit élément de support pour permettre l'observation visuelle de la séparation de corps en réaction agglutinés d'avec les corps en réaction non agglutinés.

2. Dispositif selon la revendication 1, dans lequel ladite matrice est recouverte par une solution contenant des réactifs.

3. Dispositif selon la revendication 2, dans lequel ladite solution a une densité élevée.

4. Dispositif selon la revendication 2 ou 3, dans lequel ladite solution contient des anticorps.

5. Dispositif selon la revendication 4, dans lequel les anticorps sont des anticorps IgG.
6. Dispositif selon l'une quelconque des revendications 1 à 5, dans lequel lesdites particules ont un diamètre allant de 50 à 150 μm .

7. Procédé pour la détection de la fixation de ligands, comprenant les étapes suivantes

(a) dépôt d'un échantillon susceptible de contenir un ligand, capable de se fixer, sur un dispositif selon l'une quelconque des revendications 2 à 6, dans lequel lesdits réactifs comprennent un partenaire de liaison correspondant pour ledit ligand capable de se fixer;

(b) application d'une force sur ledit échantillon, afin de provoquer le mélange dudit échantillon et de ladite solution, de manière que tout ligand capable de se fixer qui est présent dans l'échantillon se fixe à son partenaire de liaison correspondant, pour former un agglutinat, et de faire en sorte que les corps en réaction non agglutinés traversent ladite matrice et les corps en réaction agglutinés soient capturés sur ou dans ladite matrice; et

(c) détection de la présence ou de l'absence d'agglutinats au-dessus ou à l'intérieur de ladite matrice.

8. Procédé selon la revendication 7, dans lequel ladite force est appliquée par centrifugation.

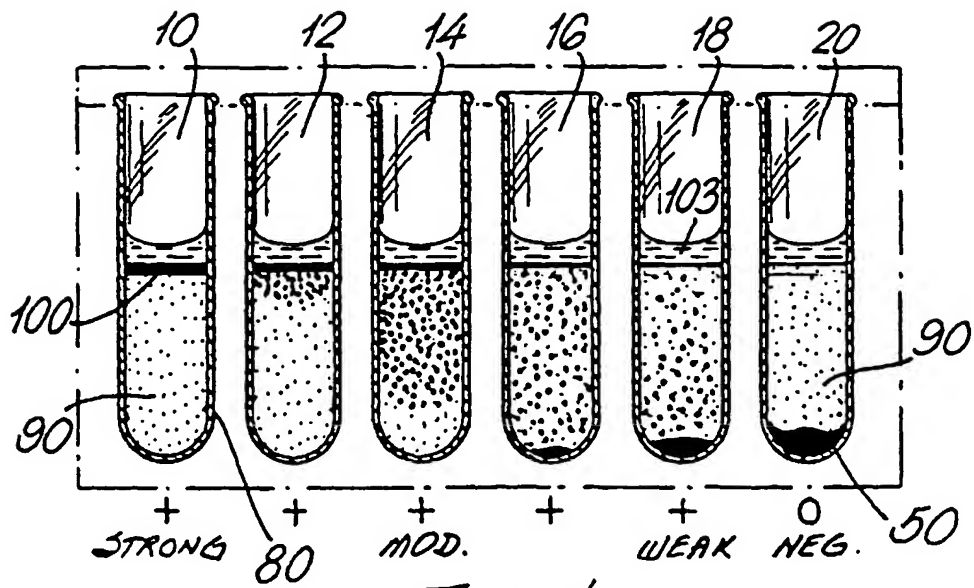


Fig. 1

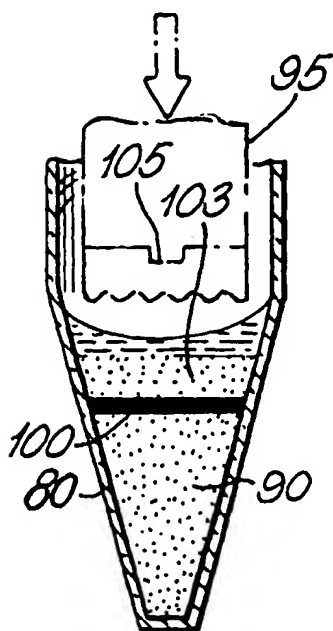


Fig. 2

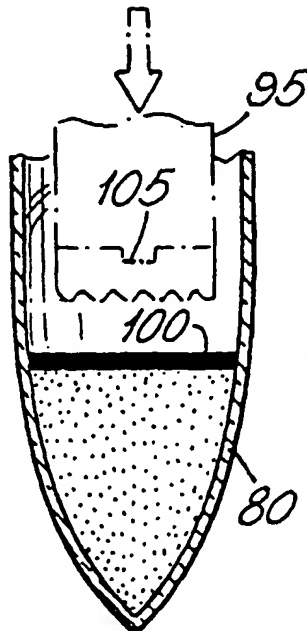


Fig. 3

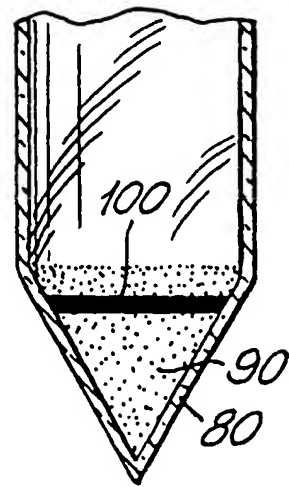


Fig. 4